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Synergic activation upon MET and ALK co-amplification sustains targeted therapy in sarcomatoid carcinoma, a deadly subtype of lung cancer

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Abstract

Hypothesis. Genetic alterations suitable for targeted therapy are poorly known issues in pulmonary sarcomatoid carcinoma (PSC), an uncommon and life-threatening family of non-small cell lung cancer.

Methods. Ninety-eight PSC were assessed for MET and ALK status by fluorescence in situ hybridization (FISH) and relevant protein expression by immunohistochemistry (IHC) also taking advantage of phosphorylated (p-) antibodies. Moreover, ALK and MET mRNA levels by real-time PCR and western blot analysis for downstream activation pathways involving p-MET, p-AKT, p-MAPK, p-SRC and p-FAK were also determined.

Results. MET amplification upon FISH was found in 25/98 (25.6%) and ALK amplification (but not the relevant rearrangement) in 16/98 (16.3%) PSC, with all ALK-amplified tumors also showing MET amplification ($p<0.0001$). Nine PSC, however, showed MET amplification without any ALK gene alteration. ALK protein expression was always lacking, while MET and p-MET were confined to the relevant amplified tumors only. Increased ALK and MET mRNA levels were detectable in tumors, with no direct relationship between mRNA content, protein expression or FISH alterations. Western blot assays showed complete activation of downstream signal pathways up to p-SRC and p-FAK recruitment in MET&ALK co-amplified tumors only, whereas isolated MET amplification, MET&ALK borderline amplification (5-10% tumor cells with ≥15 copies of the relevant gene) or negative tumors showing eusomy or chromosome polysomy were confined to p-MAPK, p-AKT and/or p-MET activation. Multivariate survival analysis pushed a higher percentage of MET altered cells or a higher value of MET copy gain per cell to marginally emerge for OS ($p=0.140$) and DFS ($p=0.060$), respectively.

Conclusions. ALK and MET seemed to act as synergic, non-random co-activators of downstream signal when co-amplified in a subset of PSC patients, thus likely suggesting a

combined mechanism of oncogene addiction. These alterations could be a suitable target for therapy upon specific inhibitors.

Introduction

Pulmonary sarcomatoid carcinoma (PSC) makes up a rare (2-3%) but deadly family of non-small cell lung carcinoma (NSCLC) encompassing pleomorphic carcinoma (PLC) (the most frequent), spindle cell carcinoma (SpCC), giant cell carcinoma (GCC), carcinosarcoma (CS) and pulmonary blastoma (PB) 1. They are deemed to be monoclonal tumor growths, in which stable and huge epithelial-mesenchymal transition (EMT) takes place causing sarcoma-like and/or sarcoma elements to arise 1-8. As sensitivity of PSC to current medical manipulations with platinum-based doublets, sarcoma-specific regimens or radiotherapy is disappointing, reverting EMT or targeting specific oncogene addiction mechanisms could be suitable therapy options for such a life-threatening tumor family 9-12. However, the lack of specific PSC-oriented clinical trials, the inherent rarity of these tumors along with troubles in their diagnostic recognition and the scarce understanding of the biological mechanisms underlying the development and progression of these tumors explain the current unsuitability for a more effective clinical management beyond surgical excision 1, 2, 11, 12. Therefore, additional genetic testing with a variety of experimental tools could allow new targets of personalized intervention to be established along with the relevant selection criteria for patients to identify. Taking into account the enormous prevalence and the social relevance of lung cancer in the human population worldwide 13, even rare tumors such as PSC are clinically meaningful at the level of individual patients.

A non-random amplification but not structural rearrangement of anaplastic lymphoma kinase (ALK) gene has recently been documented in about 18% PSC by means of fluorescence in situ hybridization (FISH) analysis 14. As no ALK protein accumulation was consistently observed upon sensitive immunohistochemistry (IHC) methods, another synergic gene driver mechanism was hypothesized to be in charge of the development and maintenance of these tumors, such as MET gene, which is recurrently altered in a subset of PSC 14, 15, with significant functional differences with lung adenocarcinoma 16-19. More recently, it has been published that mutational events of MET leading to exon 14 skipping are potentially targetable events in PSC 11 but the relevance of this mutation has been diversely reported on 12. Most importantly, the role of MET amplification and its functional interplay with ALK alterations still remains an unresolved issue.

Beyond functioning as a driver gene in the development of PSC and an adverse prognostic factor in lung cancer 20, MET is also recruited as a primary or secondary resistance mechanism to tyrosine kinase inhibitor (TKI) treatment in EGFR-mutated lung adenocarcinoma 21-24, whereas de novo MET amplification is restricted to about 3-5% of chemo-naïve adenocarcinoma patients 16, 23, 25.

This study was aimed at evaluating the status of MET and the relative interplay with ALK in a consecutive series of PSC, the largest one to be assessed thus far for either biomarker, by using several experimental approaches, such as FISH, IHC, real-time polymerase chain reaction (qPCR) and functional activation upon western blot (WB). The main goal was to offer a biological rationale to the functional utilization of MET as suitable target of therapy in these life-threatening tumors.

Patients and Methods

Overall design of the study

Cases were screened for ALK and MET gene status abnormalities by means of FISH and for the relevant expression by using enzymatic IHC, either total or phosphorylated (p) protein. Subsequently, the functional meaning of the diverse gene alterations was further untangled by addressing real-time PCR to evaluate the mRNA levels and western blot analysis to decipher the relevant downstream functional activation signals upon MET/p-MET, ALK/p-ALK, AKT/p-AKT, MAPK/p-MAPK, SRC/p-SRC and FAK/p-FAK simultaneous assessment.

Patients and tumors

A series of 98 consecutive surgical specimens of PSC from 76 males (range 38-85 years; mean ±SD 67.2±10.3) and 22 females (range 30-83 years; mean±SD 61.2±14.1) were retrieved from the pathology archives of the participant Institutions. The lack of a previous history of cancer elsewhere in the body and the availability of complete clinical information were required for entering the study. Eighty-one patients were current smokers, seven former smokers and six never smokers (smoking status was unavailable in four patients).

Surgical specimens consisted of 77 (bi)-lobectomies, 18 pneumonectomies and three segmentectomies. According to the 7th edition of the TNM staging system, there were seven tumors staged IA, 14 IB, 23 IIA, 28 IIB, 24 IIIA, one IIIB (featuring pT4pN2) and one IV (featuring pT3pN1pM1a). In detail, there were six pT1a, eight pT1b, 25 pT2a, 18 pT2b, 36 pT3 and 5 pT4 along with 57 pN0, 25 pN1 and 16 pN2 patients and a percentage of involved lymph nodes ranging from 4 to 83%. Vascular invasion was observed in 62 tumors and necrosis in 93 tumors (the latter ranging from 5% to 85% of the entire tumor mass). Pleural

invasion 26 was documented in 63 tumors resulting in 30 PL1, 11 PL2 and 22 PL3. Tumor size ranged from 3 to 150 mm, with a mean±SD value of 41.1±28.4 mm. Among patients, 14 underwent neo-adjuvant chemotherapy (for other 15 patients this information was unavailable). Follow-up information was updated to July 2015. During this period, 48 patients had recurrent disease (17 single, 18 multiple and 13 systemic relapses) and 58 died of disease, with the mean disease-free and overall survival time being 28.9±37.9 months (median 9 mo; range 1-165 mo) and 33.6±35.4 months (median 20.3 mo; range 1-165 mo), respectively. Overall data concerning the 98 PSC patients under evaluation are summed up in Table 1.

All PSC samples had been fixed in 4% buffered formaldehyde solution for 12-24 hours and embedded in paraffin according to standard histopathology methods. All the original hematoxylin and eosin stained sections were reviewed on the basis of the 2015 WHO lung cancer classification 1, without knowledge of patients' identity or original tumor categorization. Moreover, frozen samples of PSC and lung adenocarcinoma (the latter serving as control group for either MET and ALK alterations) were available for the study.

Ethics

The study was notified to and approved by the independent ethics committee of the "Fondazione IRCCS Istituto Nazionale Tumori", Milan, Italy (accession number INT-162/14). All patients gave their written consent for diagnosis and research activities upon hospital admittance.

Fluorescence in situ hybridization

Fluorescence in situ hybridization analysis was performed in 2-4 μ-thick paraffin sections by counting at least 100 tumor cells. Briefly, a commercial available break-apart, dual-color gene specific probe at 2p23 (Abbott Molecular, Vysis® LSI® ALK Dual Color, Break Apart Rearrangement Probe) was used to identify ALK alterations, whereas MET was investigated by means of a commercial available dual-color probe at 7q31 along with CEP probe for chromosome 7 centromere (ZytoLight® SPEC MET/CEN 7 Dual Color Probe, Bio-Otpica, Milan, Italy). Both probes were used according to the manufacturer's instructions, analyzing separately both the epithelial and sarcoma/sarcoma-like components on the same areas of tumor sections. For ALK, two or more red/green fusion signals (yellowish) indicated cells with wild-type configuration, whereas one or more yellowish fusion signals (wild type ALK) along with separate green and red signals, or isolated red signals, identified cells with rearranged gene. For ALK and MET amplification, quite restrictive criteria were taken into account as follows: (a) amplification, if more than 10% tumor cells presented with ≥15 copies of the relevant genes or variably sized signal clusters; (b) borderline amplification, if 5-10% tumor cells presented with ≥15 copies of the relevant gene or variably sized signal clusters; (c) eusomy, if tumor cells showed 2 to 4 signals regardless of their percentage or <5% tumor cells presented with amplification criteria (≥15 copies of the relevant gene or variably sized signal clusters) 14, 16. We preferred to use this way to express MET and ALK alterations rather than the ratio between gene and centromere signals for at least two reasons: a) different experimental conditions in the FISH analysis for MET and ALK, with dual-color assay for the former allowing simultaneous assessment of MET and chromosome 7 signals on the same tumor sections vs. break-apart probe for the latter with a separate chromosome 2 enumeration probe on adjacent tumor sections; and b) difficulty sometimes to count the exact number of MET signals clustered in dense clouds whether the relevant ratio had to be obtained.

Polysomy of chromosome 7 was assessed by CEP7 probe (centromeric alpha-satellite specific for chromosome 7, from Abbott) on the same sections as those used for MET gene evaluation, whereas an additional FISH assay was performed on adjacent sections for chromosome 2 utilizing CEP2 probe (centromeric alpha-satellite specific for chromosome 2, from Abbott). Hence, polysomy was designated as the presence of more than two specific signals for both ALK or MET and chromosome 2 or 7 centromeric probes, respectively, with a ratio ranging from 1.0 to 2.0 in the large majority of tumor cells. ALK or MET eusomy and polysomy were aggregated for statistical purposes into a unique category of negative tumors. Beyond assembling results as FISH categories, both the percentage of tumor cells harboring the relevant gene alterations (amplification, borderline amplification, negative tumors) and the mean value of signals per cell were introduced into the analysis.

Immunohistochemistry

All tumors underwent protein characterization for ALK and MET gene product by using sensitive IHC methods. Briefly, three-four micron-thick sections were unmasked with EDTA buffer at pH8 for 30 minutes and made react with the relevant antibodies (for ALK, clone 5A4, Santa Cruz, Heidelberg, Germany, dilution 1:100 for 60 min; for total MET, clone D1C2, Cell Signaling Technology, Denver, MA, USA, dilution 1:100 for 60 min; for p-MET at Tyr1234/1235, clone D26, Cell Signaling Technologies, dilution 1:50 for 30 min). Sections were then incubated with a commercially available detection kit (EnVision™ FLEX+, Dako, Glostrup, Denmark) in an automated immunostainer (Dako Autostainer System, Dako, Denmark) according to previously refined IHC methods 14. The specificity of all reactions was double-checked replacing the primary antibody with a non-related mouse immunoglobulin at a comparable dilution or using normal serum alone. Positive and negative controls were adopted as required (for ALK, a FISH-positive pulmonary adenocarcinoma carrying the specific gene rearrangement; for MET/p-MET, the alveolar rhabdomyosarcoma cell line RH30

characterized by strong reactivity 27). Results were then rendered semi-quantitatively as the percentage of labeled cells showing convincing cytoplasm labeling along with faint to strong membrane decoration on the basis of the specific protein being investigated, as previously detailed 14.

Real-time polymerase chain reaction

Total RNA from microdissected tumor samples with at least 75% cellularity was isolated by means of the TRIzol method (Life Technologies, Foster City, CA, USA) and reverse transcribed to cDNA with SuperScript® Reverse Transcriptase (Life Technologies, USA) using both oligo-dT and random hexamers. ALK and MET mRNA/cDNA were relatively quantified by real-time PCR using specific TaqMan assays (Life Technologies, USA) for the relevant gene isoforms under evaluation, as previously detailed 28. The housekeeping POL2 was used as endogenous positive control and thus amplified simultaneously with the samples. The levels of mRNA for the relevant genes were quantified in tumor samples using the 2 - ΔCT threshold method and then compared with the mRNA thresholds of non-neoplastic paired lung samples, always calibrating each case with the housekeeping gene POL2 levels. Acquisition and analysis of the data was performed using Sequence Detection System ABIPRISM 7900HY (Life Technologies, USA). All experiments were performed in triplicate for consistency.

Western blot

Cell lysate were directly separated on SDS-polyacrylamide gels. Standardized immunoblotting protocols were used to assay ALK and MET protein expression. As positive control for expression and activation of MET protein, the RH30 cell line was adopted. This cell line expresses ALK and MET protein upon IHC, as well as gene copy gain by FISH (ALK with 5-12 signals per cell and MET with 5-10 signals per cell). Two different anti-ALK antibodies

recognizing intracellular kinase-containing regions of ALK were used, namely mouse monoclonal antibody ALKc provided by Dr B. Falini, University of Perugia, Italy, and the commercially available rabbit monoclonal antibody to ALK clone D5F3 (Cell Signaling Technology®, USA). ALK activation was tested on whole lysate (100µg) using the phospho-Tyr664ALK antibody (Cell Signaling Technology®, USA). MET (D1C2) XP® rabbit monoclonal antibody and a polyclonal rabbit p-MET (Tyr1234/1235) antibody (Cell Signaling Technology®, USA) were applied to whole lysate to examine MET and its activation. Nonetheless, to observe the downstream signaling transduction activation pathways of ALK and MET, the following antibodies were applied to: AKT, p-AKT, MAPK, p-MAPK, FAK, p-FAK, SRC, p-SRC (all from Cell Signaling Technology, ® USA). Furthermore, VINCULIN (Cell Signaling Technology®, USA) was assayed as positive control for protein loading.

Statistical analysis

Qualitative data were compared by Fisher's exact probability test or chi-square test as appropriate, whereas continuous data were contrasted with Mann-Whitney test. The strength of association between variables showing quantitative categories of unequal magnitude (i.e. MET and ALK gene FISH alterations) was evaluated by using contingency coefficient and Cramer's V (ϕc), which may vary between 0 (independence or no association) and 1 (dependence or complete association). Overall survival (OS) was defined as the time between surgery and the last follow-up or cancer death. If a patient died without cancer recurrence, the patient's survival time was censored at the time of death. Only lung cancer-related deaths or recurrences were considered to be events. Disease-free survival (DFS) was calculated from the date of surgery to the date of progression or the date of last follow-up. Survival estimates were calculated with Kaplan-Meier's method and compared by the log rank test. The Cox proportional hazard regression model was used to evaluate the simultaneous effect of explanatory variables on survival time. All analyses were carried-out

using the SAS statistical software (SAS Institute, Inc., Cary, NC, USA). For all tests, only two-sided p-values were taken into account (with a threshold of <0.05 for statistical significance) and confidence intervals set at the 95% level.

Results

Pathology

The study comprised 83 PLC, 5 PB, 4 GCC, 3 CS and 3 SpCC. Out of 83 PLC, 73 tumors showed an epithelial cell component, whereas ten were composed of sarcoma-like elements only featuring spindle and giant cells. Out of these 73 tumors, 53 included adenocarcinoma ranging from to 10 to 90%, 15 squamous cell carcinoma ranging from 10 to 85%, and 5 adenosquamous carcinoma ranging from 40 to 80%. In the same tumor subtype, the sarcoma-like component accounted for spindle cells in 24 and giant cells in nine tumors (either ranging from 15 to 95%), whereas a combination of spindle and giant cells were observed in 50 tumors to range from 10 to 100%.

In PB, the epithelial component comprised well-differentiated fetal adenocarcinoma (ranging from to 20 to 80%), while the sarcomatous component featured spindle cells of adult type in 2 tumors and blastematous cells with rhabdomyosarcoma elements in 3 tumors (ranging from 20 to 80%).

In CS, the epithelial component was adenocarcinoma in two cases and squamous cell carcinoma in one case, averaging 30% of tumor mass, whereas the sarcoma component was rhabdomyosarcoma, osteosarcoma or a combination of either tumor type (ranging from 70 to 90%).

In GCC and SpCC there was an exclusive component of giant or spindle cells, respectively (Figure 1 A and 1 D). Lymph node metastases were of epithelial type in 25 tumors and sarcoma-like in 16 tumors. Apart from tumor necrosis amount, no other relationship was noted between tumor stage (dichotomized as stage I vs. II-IV) and histological classification, tumor cell composition and vascular invasion (Supplemental Material A).

Distribution of MET and ALK alterations

Gene amplification, borderline amplification, eusomy status and chromorome 2 or 7 polysomy were identified in 25 (25.6%), 6 (6.1%), 16 (16.3%) and 51 (52.0%) tumors and 16 (16.3%), 9 (9.2%), 22 (22.5%) and 51 (52.0%) tumors for MET and ALK, respectively. No ALK rearrangement was observed in the tumor series under evaluation. MET amplification was mostly characterized by variably sized clusters up to clouds of signals as opposed to discrete single signals per tumor cell for ALK (Figure 1 C and 1 F). A close relationship was noticed between MET and ALK amplification, with all 16 ALK-amplified tumors being MET co-amplified in the same relevant tumors cells that in turn also included three ALK-borderline amplified and six ALK-negative tumors ($p<0.0001$) (Table 2). The strength of this association was confirmed by contingency coefficient and Cramer's V (ϕc) values of 0.72. All the cases showing MET or ALK FISH amplification also presented with higher vales of the relevant ratios between gene and centromere signals (data not shown).

As far as the percentage of altered tumor cells was concerned, the mean value was 57.5±30.5% (range 11% to 69%) for MET-amplified tumors (22 PLC, two PB and one CS) and 45.0±26.4% (range 11%-43%) for ALK-amplified tumors (14 PLC and two PB) ($p=0.0032$), while the number of signals per cell was on average 4.9±2.1% for MET and 4.3±1.6% for ALK ($p=0.0357$). No differences in MET and ALK alterations were found between epithelial and sarcoma-like/sarcoma elements. For MET but not ALK, the relevant amplification was marginally associated with advanced stage (II-IV) of disease ($p=0.080$) and an increased percentage of altered cells ($p=0.060$). A significant association was documented for MET-amplified or borderline-amplified tumors with spindle and giant cell component ($p=0.046$), whereas no associations were seen for ALK with the percentage and the type of tumor cells.

Protein expression

MET (Figure 1 B) and p-MET (Figure 2 A) expression was present as membrane decoration in most tumor cells of all MET-amplified and MET-borderline amplified tumors, whereas fainter to negative decoration was observed in the tumors with eusomy for the relevant gene or chromosome 7 polysomy. MET or p-MET membrane labeling was mainly confined to elements featuring epithelial cells, while sarcoma-like/sarcoma elements were either negative or exhibited vanishing membrane decoration only. In turn, ALK protein was always negative regardless of the type of concurrent gene alteration (Figure 1 E).

mRNA expression

Real-time PCR examination, carried out in 22 PLC and one SpCC, showed detectable levels of MET mRNA in six MET-amplified, one MET-borderline amplified and 16 negative tumors, while only six tumors revealed analyzable thresholds of ALK mRNA compared to non-neoplastic lung parenchyma (Supplemental Material B). No direct relationship was observed for either gene between mRNA levels and protein expression, FISH alterations or clinico-pathological variables.

Biochemical assays

Biochemical investigation was feasible on 8 PSC, which frozen material was available for, with results being depicted in Figure 2 B. As positive control, the RH30 cell line showing gene copy gain for ALK and MET was adopted (lane 1). Out of the 8 tumor samples under evaluation, lane 2 refers to isolated MET amplification, lanes 3-5 to MET and ALK simultaneous co-amplification, lane 6 to MET and ALK borderline co-amplification and lanes 7 through 9 to negative tumors (eusomy or chromosome 7 or 2 polysomy) for either gene.

Interestingly, while ALK total protein or p-ALK was never documented in our cases, downstream activation was seen in lines 2 to 6 with bands for p-MET, p-AKT and p-MAPK in MET-positive cases, which comprised isolated MET amplification, MET&ALK co-amplification and MET&ALK borderline co-amplification. However, there were worthwhile differences among these tumors, inasmuch as faint to strong p-SRC and p-FAK signals were present only in cases exhibiting simultaneous MET&ALK co-amplification. In turn, p-AKT and p-MAPK were recognizable even in not amplified tumors for either MET or ALK, which failed to show signals for p-MET, p-SRC and p-FAK (lanes 7 to 9). Interestingly, there was a prevalence of sarcoma-like/sarcoma cells in tumors expressing MET&ALK co-amplification or borderline amplification over tumors with MET amplification alone, which showed a prevalence of epithelial cells ($p=0.0001$).

Clinical correlations

Overall survival was assessable in 92 patients and DFS in 72 patients. No association was seen for OS and DFS with ALK&MET co-amplification as opposed to borderline amplification and negative tumors (Supplemental Material C). A marginally significant shorter OS but not DFS was documented in tumors expressing higher percentage of MET-altered cells ($p=0.064$), whatever type of alterations were dealing with, according to the median value of 61%. Other OS determinants were pN status ($p<0.0001$), the percentage of metastatic LN ($p=0.008$) and, marginally, vascular invasion ($p=0.076$) and the percentage of tumor necrosis ($p=0.106$). Disease-free survival was affected by pN status ($p=0.028$), the percentage of metastatic LN ($p=0.006$), the percentage of tumor necrosis ($p=0.069$) and, marginally, MET average signals per cell according to the median value of 4.4 ($p=0.052$). No survival relationship was seen with any ALK gene alteration. Patients undergoing neo-adjuvant chemotherapy differed marginally from untreated patients for DFS ($p=0.074$) but not OS or other bio-pathological variables. Multivariate survival models pushed pN status ($p=0.002$)

and, marginally, higher median percentage of MET-altered cells (p=0.120) to emerge for OS and pN status (p=0.010) and, marginally, tumor necrosis (p=0.130) and higher median value of MET copy gain per cell (p=0.070) to emerge for DFS. Notably, tumor stage was did not independently affect survival (see Table 3 for details).

Discussion

The main findings of our investigation are that MET amplification is present in a subset of PSC and is a potential valuable target for therapy options in a currently orphan tumor with ineffective treatments other than surgical excision Accordingly, MET amplification may be really an Achilles heel in this clinical setting of tumors, inasmuch as it may be effectively targeted by specific tyrosine kinase inhibitors.

Our investigation displayed a very close correlation between MET and ALK alterations upon FISH analysis, with about one fifth of PSC showing co-amplification for both genes, as also confirmed by high thresholds of contingency coefficient and Cramer's V statistics. In turn, no ALK rearrangement was observed in PSC as previously reported 14. An immediate contention is that these cytogenetic aberrations are non-random events in PSC, where are likely to play addiction mechanisms to the development and maintenance of at least a subset of them. While ALK rearrangement was never observed and MET mutation not evaluated in the present study as recently pointed out 11, 12, we noted that MET-amplified tumors prevailed over ALK-amplified tumors as either the number of altered cases or the amount of involved tumor cells, in both epithelial and sarcoma-like/sarcoma components. These data sustained not only a driver role of MET amplification in the genesis of a few PSC, but also suggested a non-negligible and causative effect of concurrent ALK as co-actor despite undetectable protein accumulation even in amplified and borderline-amplified tumors. However, experiments conducted by exploiting very sensitive immunofluorescence techniques revealed that there was faint accumulation of ALK protein in the cytoplasm of ALK-amplified but not borderline-amplified tumors, which failed however to be demonstrated with bright-field IHC or western blot analysis probably due to the higher sensitivity of the immunofluorescence approach (data not shown). As a matter of fact very low levels of ALK mRNA were otherwise detected by means of real-time PCR as opposed to complete absence in normal lung parenchyma of the same patients.

Furthermore, we confirmed our previous observations 14 that ALK or MET amplification was closely associated with chromosome 7 or chromosome 17 polysomy but neither EGFR nor Her-2/neu amplification, thus minimizing the risk of facing with pan-amplification linked to clonal selection, tumor progression or chromosome instability of highly malignant lesions (data not shown). The functional role of MET as driver mechanism in a few PSC was also endorsed by the prevalence of total MET- and p-MET-protein accumulation in amplified and borderline amplified tumors, while negative lesions (bearing eusomy or polysomy status) turned out faintly decorated for total MET but consistently negative for p-MET, suggesting the presence of only functionally inactive receptor in these tumors.

The magnitude of MET amplification intended as the higher percentage of amplified cells in amplified and borderline-amplified cases compared to the relevant ALK recruitment indicated that MET amplification was the driver event in this subset of PSC, while ALK amplification occurred as a second hit to strengthen the biological mechanism of this gene alteration. This hypothesis was further reinforced by the observation that, out of nine PSC identified as bearing ALK-borderline amplification, there were three MET amplified and five MET borderline-amplified tumors, with only one case bearing eusomy for the relevant gene alteration. On the contrary, out of six MET-borderline amplified tumors, there were one ALK-negative and five ALK borderline amplified tumors.

No direct relationship was documented between mRNA content and protein expression or any FISH alteration for both MET and ALK, suggesting some post-transcriptional and non-linear regulation mechanisms. While MET membrane expression, along with darker cytoplasm decoration was present in the majority of cases regardless of their cytogenetic alteration, p-MET IHC paralleled functional biochemistry results indicating that only those cases with MET gene amplification were also positive for p-MET by IHC, whereas cases without the protein activation turned out to be completely negative. Interestingly, in the specific cases with MET amplification, p-MET IHC was stronger in epithelial cells while

decreased in intensity and vanished as soon as sarcoma-like/sarcoma cell status were reached. This finding was in keeping with the assumption that MET is one of the actors playing a role in EMT occurring in lung cancer, which stably characterizes PSC 2. In this respect, only MET&ALK co-amplification turned out in multi-signal activation cascade including p-SRC and p-FAK beyond p-MET, p-AKT and p-MAPK, which are common to both ALK and MET downstream pathways leading to increased cell motility, invasion ability and EMT occurrence 29, 30. Conversely, isolated MET amplification or MET&ALK borderline co-amplification (all our ALK-amplified tumors were also MET-amplified) resulted in MET activation (with p-MET upon IHC and biochemical assays) along with p-AKT and p-MAPK only. Furthermore, there was a significant relationship between MET&ALK co-amplification and the prevalence of sarcoma-like/sarcoma elements in the relevant tumors ($p=0.0001$), supporting once again a causative role of both genes in sustaining mechanisms of EMT in a subset of PSC. Our findings support the view that other factors were likely to play an active role in inducing EMT in PSC when lacking complete MET&ALK co-amplification. Tumors harboring eusomy for MET or ALK or polysomy for the relevant chromosome exhibited p-AKT and p-MAPK but not p-MET (p-ALK was never seen in functional tests), indicating that different mechanisms of EMT occurred in PSC. The close co-linearity between MET and ALK aberrations pushed the gene copy gain to emerge as causative inducer of the type of signal cascade being activated. In this regard, a MET&ALK copy number gain consistent with amplification caused SRC and FAK pathway to recruit, whilst lower gene copy gain failed to activate the same pathway effectively. Interplaying between different oncogenes due to co-activation is not surprising in lung cancer and may define distinct subsets of tumors with different biological properties and clinical outcome 31.

Clinical correlations showed that MET but not ALK FISH alterations were weak prognostic factors, whereas the value of our observations regarded the opportunity to use MET and ALK as potential co-targets of therapy. Recent data stemming from preclinical analyses and early-

phase clinical trials in NSCLC other than PSC have demonstrated that ALK and MET inhibitors specifically reduced proliferation in tumors carrying either rearrangement or amplification, and that MET protein accumulation and gene amplification were predictors of response in NSCLC to the treatment with MET monoclonal antibodies or tyrosine kinase inhibitors 32. There are no systematical data on the predictive role of ALK amplification in lung cancer, but NSCLC cells driven by ALK amplification and oncogenic rearrangements of ROS1 and RET kinase genes have been shown to be sensitive to ganetespib exposure, a Hsp90 inhibitor 33. This reinforces once again the notion that ALK amplification could be the tip of the iceberg of other underlying gene alterations, which in our study involved MET gene in a subset of tumors.

Mutational events of MET have recently been reported to occur in PSC 11, 12, but results are conflicting about the relative prevalence of mutations ranging from less than 5% 12 to 22% 11, hence revealing an unexpected inter-tumor heterogeneity likely due to geography, ethnicity and gender issues or tumor composition. Few papers have thus far investigated the causative relationship between MET or ALK gene mutations and copy number variations, but this association seems unlikely to occur in diverse sets of tumors34, 35. As these events could even point to different cohorts of patients, it is important for biological and clinical reasons to compare the mutational burden of PSC by using high-throughput methods, such as next generation sequencing, with the underlying copy number variation by means of reproducible techniques, such as FISH analysis (Pelosi et al, manuscript in preparation). As a matter of fact establishing the relationship between gene amplification and mutational status may provide meaningful information on secondary resistance mechanisms while using tyrosine kinase inhibitors.

Conclusions

We herein have documented a likely causative co-activation of MET and ALK in the development and maintenance of a subset of PSC and validated FISH as the best methodology to test this particular subgroup of tumors. These findings pave the way to the clinical use of targeted therapy based on specific pathway inhibitors.

Figure legends

Figure 1 A-F. Pulmonary sarcomatoid carcinoma of giant cell type is shown (A), which was positive for MET protein (B) and exhibited MET gene amplification as indicated by the presence of over 15 copies of fluorescence signal per cell in more than 10% of tumor cells (C). Another example of pulmonary sarcomatoid carcinoma of pleomorphic cell type comprising spindle and giant cells is depicted (D), showing complete absence of ALK protein (E) and a distinct signal pattern indicating ALK gene amplification (F).

Figure 2 A-B. Pulmonary sarcomatoid carcinoma of pleomorphic type with positive p-MET immunohistochemistry in the epithelial cell component is shown, whereas adjacent sarcoma-like elements are negative (A). The panel of western blot analysis is presented according to diverse lanes corresponding to different tumor categories. At variance with MET-amplified tumors (lane 2), MET&ALK borderline amplified tumors (lane 6) and negative tumors with eusomy or chromosome polysomy (lanes 7 to 9), tumors exhibiting concurrent MET and ALK amplification presented with different pathways of activation involving p-SRC and p-FAK. This indicated that it was the entity of gene copy gain for MET and ALK to affect the ultimate functional modality of activated downstream signals. RH30 rhabdomyosarcoma cell line is shown in lane 1, which exhibited positive ALK and MET immunohistochemistry and gene copy gain for ALK (5-12 signals) and MET (5-10 signals) (B).

Supplemental Material A. Pathological results organized according to tumor stage. Only necrosis amount was significantly associated with more advanced tumor stage (p=0.003).

Supplemental Material B. Real-time PCR analysis results for MET and ALK according to FISH results and some clinico-pathologic variables. No direct relationship was observed for either gene between mRNA levels and protein expression, FISH alterations or clinico-pathological variables.

Supplemental Material C. Overall or disease-free survival was not affected by MET and ALK FISH co-amplification as contrasted to borderline amplification and negative tumors.

References

1. Travis W, Brambilla E, Burke A, Marx A, Nicholson A. WHO Classification of Tumours of the Lung, Pleura, Thymus and Heart. Lyon: IARC Press; 2015.

2. Pelosi G, Sonzogni A, De Pas T, et al. Review article: pulmonary sarcomatoid carcinomas: a practical overview. Int J Surg Pathol 2010;18:103-20.

3. Rossi G, Cavazza A, Sturm N, et al. Pulmonary carcinomas with pleomorphic, sarcomatoid, or sarcomatous elements: a clinicopathologic and immunohistochemical study of 75 cases. Am J Surg Pathol 2003;27:311-24.

4. Pelosi G, Fraggetta F, Nappi O, et al. Pleomorphic carcinomas of the lung show a selective distribution of gene products involved in cell differentiation, cell cycle control, tumor growth and tumor cell motility: a clinicopathological and immunohistochemical study of 31 cases. Am J Surg Pathol 2003;27:1203-15.

5. Blaukovitsch M, Halbwedl I, Kothmaier H, Gogg-Kammerer M, Popper HH. Sarcomatoid carcinomas of the lung--are these histogenetically heterogeneous tumors? Virchows Arch 2006;449:455-61.

6. Cates JM, Dupont WD, Barnes JW, et al. Markers of epithelial-mesenchymal transition and epithelial differentiation in sarcomatoid carcinoma: utility in the differential diagnosis with sarcoma. Appl Immunohistochem Mol Morphol 2008;16:251-62.

7. Dacic S, Finkelstein S, Sasatomi E, Swalsky P, Yousem S. Molecular pathogenesis of pulmonary carcinosarcoma as determined by microdissection-based allelotyping. Am J Surg Pathol 2002;26:510-6.

8. Lee JM, Dedhar S, Kalluri R, Thompson EW. The epithelial-mesenchymal transition: new insights in signaling, development, and disease. J Cell Biol 2006;172:973-81.

9. Bae HM, Min HS, Lee SH, et al. Palliative chemotherapy for pulmonary pleomorphic carcinoma. Lung Cancer 2007;58:112-5.

10. Kaira K, Horie Y, Ayabe E, et al. Pulmonary pleomorphic carcinoma: a clinicopathological study including EGFR mutation analysis. J Thorac Oncol 2010;5:460-5.

11. Liu X, Jia Y, Stoopler MB, et al. Next-Generation Sequencing of Pulmonary Sarcomatoid Carcinoma Reveals High Frequency of Actionable MET Gene Mutations. J Clin Oncol 2015

12. Fallet V, Saffroy R, Girard N, et al. High-throughput somatic mutation profiling in pulmonary sarcomatoid carcinomas using the LungCarta Panel: exploring therapeutic targets. Ann Oncol 2015;26:1748-53.

13. Torre LA, Bray F, Siegel RL, et al. Global cancer statistics, 2012. CA Cancer J Clin 2015;65:87-108.

14. Pelosi G, Gasparini P, Cavazza A, et al. Multiparametric molecular characterization of pulmonary sarcomatoid carcinoma reveals a nonrandom amplification of anaplastic lymphoma kinase (ALK) gene. Lung Cancer 2012;77:507-14.

15. Lee S, Kim Y, Sun JM, et al. Molecular profiles of EGFR, K-ras, c-met, and FGFR in pulmonary pleomorphic carcinoma, a rare lung malignancy. J Cancer Res Clin Oncol 2011;137:1203-11.

16. Pelosi G, Gasparini P, Sozzi G, et al. Fluorescence in situ hybridization (FISH)-assessed amplification of anaplastic lymphoma kinase (ALK) gene is detecable in a subset of pulmonary sarcomatoid carcinomas (PSC) (Abstract # 2027). Mod Pathol 2012;25 (Suppl 2):486A.

17. Tanizaki J, Okamoto I, Sakai K, Nakagawa K. Differential roles of trans-phosphorylated EGFR, HER2, HER3, and RET as heterodimerisation partners of MET in lung cancer with MET amplification. Br J Cancer 2011;105:807-13.

18. Tanizaki J, Okamoto I, Okamoto K, et al. MET tyrosine kinase inhibitor crizotinib (PF-02341066) shows differential antitumor effects in non-small cell lung cancer according to MET alterations. J Thorac Oncol 2011;6:1624-31.

19. Nakamura Y, Niki T, Goto A, et al. c-Met activation in lung adenocarcinoma tissues: an immunohistochemical analysis. Cancer Sci 2007;98:1006-13.

20. Raghav KP, Gonzalez-Angulo AM, Blumenschein GR, Jr. Role of HGF/MET axis in resistance of lung cancer to contemporary management. Transl Lung Cancer Res 2012;1:179-93.

21. Tsuta K, Kalhor N, Wistuba, II, Moran CA. Clinicopathological and immunohistochemical analysis of spindle-cell carcinoid tumour of the lung. Histopathology 2011;59:526-36.

22. Tsuta K, Kozu Y, Mimae T, et al. c-MET/phospho-MET protein expression and MET gene copy number in non-small cell lung carcinomas. J Thorac Oncol 2012;7:331-9.

23. Ou SH, Kwak EL, Siwak-Tapp C, et al. Activity of crizotinib (PF02341066), a dual mesenchymal-epithelial transition (MET) and anaplastic lymphoma kinase (ALK) inhibitor, in a non-small cell lung cancer patient with de novo MET amplification. J Thorac Oncol 2011;6:942-6.

24. Sequist LV, Waltman BA, Dias-Santagata D, et al. Genotypic and histological evolution of lung cancers acquiring resistance to EGFR inhibitors. Sci Transl Med 2011;3:75ra26.

25. Sgambato A, Casaluce F, Maione P, et al. The c-Met inhibitors: a new class of drugs in the battle against advanced nonsmall-cell lung cancer. Curr Pharm Des 2012;18:6155-68.

26. Travis WD, Brambilla E, Rami-Porta R, et al. Visceral pleural invasion: pathologic criteria and use of elastic stains: proposal for the 7th edition of the TNM classification for lung cancer. J Thorac Oncol 2008;3:1384-90.

27. Taulli R, Scuoppo C, Bersani F, et al. Validation of met as a therapeutic target in alveolar and embryonal rhabdomyosarcoma. Cancer Res 2006;66:4742-9.

28. Ciniselli CM, Volpi CC, Cortelazzi B, et al. Reproducibility between messenger RNA real-time polymerase chain reaction and messenger RNA in situ hybridization in patients with oropharyngeal squamous cell carcinoma. Hum Pathol 2015

29. Eder JP, Vande Woude GF, Boerner SA, LoRusso PM. Novel therapeutic inhibitors of the c-Met signaling pathway in cancer. Clin Cancer Res 2009;15:2207-14.

30. Lim MS. Unraveling ALK signaling through phosphoproteomics. Blood 2009;113:2615-6.

31. Matsubara D, Ishikawa S, Sachiko O, et al. Co-activation of epidermal growth factor receptor and c-MET defines a distinct subset of lung adenocarcinomas. Am J Pathol 2010;177:2191-204.

32. Matsubara D, Ishikawa S, Oguni S, et al. Molecular predictors of sensitivity to the MET inhibitor PHA665752 in lung carcinoma cells. J Thorac Oncol 2010;5:1317-24.

33. Sang J, Acquaviva J, Friedland JC, et al. Targeted inhibition of the molecular chaperone Hsp90 overcomes ALK inhibitor resistance in non-small cell lung cancer. Cancer Discov 2013;3:430-43.

34. Lee J, Ou SH, Lee JM, et al. Gastrointestinal malignancies harbor actionable MET exon 14 deletions. Oncotarget 2015;6:28211-22.

35. Zenali M, deKay J, Liu Z, et al. Retrospective Review of MET Gene Mutations. Oncoscience 2015;2:533-41.